Lymphoproliferative assay

Harvested splenic lymphocytes were pooled for two mice in each immunized group and suspended to a concentration of 5 x 10^6 cells/ml. A 100 µl aliquot, containing 5 x 10^5 cells, was immediately added to each well of a 96-well, flat bottom microtiter plate. Reconstituted peptide, *in vitro* translated protein, or *in vitro* translation control were added to the wells, at concentrations of 5 µg/ml and 1 µg/ml (and 0.5 µg/ml for *in vitro* translated protein and *in vitro* translation control protein). Concanavalin A (Con A) was used as a positive proliferation control. The assay conditions were set up in triplicate. The cells were incubated at 37°C in 5% CO_2 for three days. One µCi of tritiated thymidine was added to each well and the cells were incubated for 18 hours at 37°C. The plate was harvested and the amount of incorporated tritiated thymidine was measured in a Beta Plate reader (Wallac, Turku, Finland). Stimulation Index was determined from the formula:

Stimulation Index (SI) = (experimental count / spontaneous count).

Spontaneous count wells included 5 % fetal bovine serum which served as an irrelevant protein control. The results are presented in Table 1.

Splenocytes isolated from mice immunized with either pWNVy-DJY or pWNVh-DJY ("H" or "Y") and incubated with WNVC-P3 ("Peptide 3") or a mixture of all three Cp peptides ("Peptide 123") yielded SI values significantly higher than did splenocytes isolated from mice in the group immunized with the base vector pcDNA3.1.

Table 1

Antigen or Stimulus	Splenocyte Source	Concentration of Protein or Peptide		
		5 μg/ml	1 μg/ml	0.5 μg/ml
Peptide 1	H	0.5	0.7	
	Y	0.8	1.2	
	pcDNA3.1	0.9	1.3	
Peptide 2	H	1.7	1.3	
	Y	1.6	1.8	
	pcDNA3.1	0.9	1.0	
Peptide 3	H	1.5	2.0	
	Y	1.1	1.4	
	pcDNA3.1	0.8	0.7	
Peptide 123	H	2.6	3.9	
	Y	1.8	2.1	
	pcDNA3.1	0.7	1.2	
Y protein	H	0.0	0.8	1.7
	Y	0.0	0.6	1.7
	pcDNA3.1	0.0	1.2	1.2
Ctrl pro	H	0.0	0.5	2.7
	Y	0.3	0.4	1.8
	pcDNA3.1	3.8	1.8	1.9
Con A	H	686.5		
	Y	366.9		
	pcDNA3.1	71.8		

Table 1 presents the results of the lymphoproliferation assay. The values presented for each condition are stimulation indices averaged over triplicate wells. For each immunization group tested, splenocytes were pooled from two mice within the group. "H" indicates splenocytes from the pWNVh-DJY-immunized group. "Y" indicates splenocytes from the pWNVh-DJY-immunized group of mice. "pcDNA3.1" indicates splenocytes from the pcDNA3.1-immunized control group of mice. Peptides 1, 2, and 3 are the WNVC-P1, WNVC-P2, and WNVC-P3 peptides described above. "Peptide 123" indicates a mixture of peptide 1, 2, and 3. "Y protein"

indicates the Cp protein *in vitro* translated from the pWNVy-DJY construct. "Ctrl pro" indicates the *in vitro* translation control, generated with pcDNA3.1 vector containing no expressible insert, as described above.

Detection of intracellular IFN-γ by flow cytometry

100 µl RPMI-1640, supplemented with 5 % fetal bovine serum (FBS) (R5 medium), containing 50 U/ml recombinant human interleukin-2 (rHuIL-2) (Intergen, Purchase, NY), 10 µg/ml brefeldin A (BD PharMingen, San Diego, CA), 100 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma, St. Louis, MO), and 1 µg/ml ionomycin (Sigma), was added to each well of a round-bottom 96-well plate. *In vitro* translated Cp protein or *in vitro* translated control protein, at 4 µg/ml, was added in 50 µl of R5 medium. After adding the protein antigens (Ag), isolated splenocytes were added to each well at $1x10^6$ cells in 50 µl of R5 medium. For the compensation in flow cytometry, splenocytes from naïve mice were incubated with only IL-2 and brefeldin A. The plates were incubated in a 37°C, 5% CO_2 incubator for 5 to 6 hours. As a control, splenocytes were also incubated without Ag. After incubation, plates were spun at 1200 rpm for 5 minutes and supernatants were discarded. The cells in each well were resuspended with 200 µl of PBS, supplemented with 1% bovine serum albumin (BSA), put on ice for 15 minutes, spun at 1200 rpm, and resuspended with in 50 µl PBS/1% BSA containing 0.1 µg of PE-conjugated, anti-CD4 mAb and 0.1 µg CyC-conjugated anti-CD44 mAb (both from BD PharMingen, San Diego, CA). After incubating for 30 minutes at 4°C, the cells were washed twice with PBS/1% BSA, the cell pellets were resuspended with 100 µl of Cytofix/Cytoperm solution (BD PharMingen, San Diego, CA), and incubated for 20 minutes at 4°C. The cells were washed twice with 1 x Perm/Wash (BD PharMingen, San Diego, CA), and resuspended with 50 µl of Perm/Wash solution containing allophycocyanin (APC)-conjugated anti-IFN-γ antibody (BD PharMingen, San Diego, CA) at 0.1 µg/sample concentration. After incubation for 30 minutes at 4°C, the cells were washed twice with 1 x Perm/Wash solution and fixed with 2% paraformaldehyde and stored at 4°C until being analyzed by flow cytometry.

CD44 expression is used as an activation marker. CD44 is a cell adhesion receptor, widely expressed on hematopoietic and non-hematopoietic cells. BALB/c mice have relatively large subsets of CD44H+ T cells. In the periphery, the level of CD44 expression increases upon activation of B cells, CD4+ T cells, CD8+ T cells, and memory cells, which can be identified by their CD44hi phenotype (expressing high levels of CD44H isoform).